

1 **Chemical degumming increases larvae size and facilitates the**
2 **commercial production of Lumpfish (*Cyclopterus lumpus*) eggs**

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8

9 **Abstract**

10 Many fishes produce adhesive eggs that confer protection from currents and
11 predators in the wild, but that are more difficult to disinfect and aerate under
12 aquaculture conditions. Removing egg adhesiveness ('degumming') has proved
13 beneficial in the culture of many fish, and a recent gap analysis identified this as a
14 potential way of increasing hatching success and minimize the risk of infectious
15 diseases in the culture of lumpfish (*Cyclopterus lumpus*), a novel species to
16 aquaculture. We tested the efficacy of the enzyme alcalase (0.02%, 0.2%, 2%) as a
17 degumming agent for lumpfish eggs, and examined its effects on hatching success,
18 survival, and larvae size under laboratory and commercial conditions. A five-minute
19 exposure to 0.2% and 2% alcalase decreased chorion thickness by 14% and
20 resulted in 61-75% degumming rates, without any negative effects on hatching rate,
21 larval survival, or incidence of embryo malformations. Degummed eggs hatched
22 earlier than controls and resulted in larger larvae, which may confer some benefits
23 under aquaculture conditions. A cost-benefit analysis indicates that the benefits of
24 egg degumming compensate the costs of chemical treatment under most conditions,
25 and that the optimal alcalase concentration is around 0.2%. We therefore
26 recommend egg degumming as a way of making the lumpfish industry more efficient
27 and sustainable.

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29 **Statement of relevance:** Improving the commercial production of lumpfish

30

31 **Keywords:** Adhesive eggs, Alcalase, Cleaner fish, Cost benefit, Egg degumming,
32 Hatching

33

34 **1. Introduction**

35 Many fish species lay eggs that become adhesive in contact with the water, which is
36 thought to be adaptive as it increases embryo survival (Rizzo et al., 2002). For
37 example, some freshwater fish produce sticky eggs that cling to the substrate to
38 avoid being washed away with the current, while other species may produce
39 adhesive eggs to form discrete clumps that are easier to tend, or that stick to the
40 vegetation to decrease predation risk or increase oxygen supply (Riehl &
41 Patzner, 1998). However, the same adhesiveness that benefits eggs in the wild may
42 hamper their viability under aquaculture conditions, where eggs are typically reared
43 at very high densities and, if untreated, are prone to suffer from fungal infections and
44 other diseases (Ringle et al, 1992).

45 The benefits of removing the adhesiveness of the eggs ('degumming') in fish
46 farming have long been known (Rottman et al., 1991; Billard et al. 1995). These
47 include improved aeration, reduced risk of fungal diseases, more effective
48 disinfection, and less effort associated with egg count and husbandry (Ringle et al,
49 1992). Egg degumming has proved beneficial for the culture of many freshwater
50 fishes, including cyprinids (Linhart et al, 2003; El-Gamal & El-Greisy, 2008; Žibienė
51 et al., 2017), tench (*Tinca tinca*, Linhart et al., 2000; Gela et al., 2003; Kujawa et al.,
52 2010), walleye (*Sander vitreus*, Krise et al., 1986), pikeperch (*Sander lucioperca*,
53 Demska-Zakes et al., 2005), barbels (Al Hazzaa & Hussein, 2003), sturgeons
54 (Pšenička, 2016), and catfishes (Isaac & Fries, 1991; Ringle et al., 1992; Linhart et
55 al., 2003; Asraf et al., 2013; Muchlisin et al., 2014; Kareem, et al., 2017). Although
56 less common in marine aquaculture, egg degumming has also proved effective for
57 anadromous and marine species (white sturgeon, *Acipenser transmontanus*; Kowtal
58 et al, 1986; ballan wrasse, *Labrus bergylta*; Grant et al., 2016).

59 Naturally sticky fish eggs have been chemically treated with many different
60 degumming agents with various degrees of success, including urea (Kowtal et al.,
61 1986), milk (Billard et al., 1995; Linhart et al., 2000), pineapple juice (Thai & Ngo,
62 2004), clay (Siddique et al., 2016), talc (Linhart et al. 2002), tannic acid (Demska-
63 Zakes et al., 2005), sodium chloride (Kowtal et al., 1986) and sodium sulphite (Isaac
64 & Fries, 1991) among others. Glycoproteins and glycosaminoglycans present in the
65 egg chorion are the molecules responsible for making fish eggs sticky (Riehl &
66 Patzner, 1998), and for this reason enzymes that can break down peptide bonds,
67 such as papain (Isaac & Fries, 1991), trypsin (Linhart et al., 2002) and various endo-
68 proteases (Linhart et al., 2003; El-Gamal & El-Greisy, 2008), have proved
69 particularly effective as degumming agents. Among these, alcalase (a commercial
70 endo-protease with broad specificity obtained from the bacterium *Bacillus*
71 *licheniformis*) has been effective in both freshwater (Linhart et al., 2003) and marine
72 species (Grant et al., 2016). However, egg degumming also poses risks and could
73 easily damage the fragile fish embryo (Pšenička, 2016). Given that species differ
74 markedly in the structure and chemical composition of the egg chorion (Riehl &
75 Patzner, 1998), no single degumming method is likely to be suitable in all cases.

76 One novel species to Aquaculture that has adhesive eggs and whose
77 incubation in hatcheries could benefit from egg degumming is the lumpfish,
78 *Cyclopterus lumpus* (Powell et al., 2018a). Large scale commercial production of
79 lumpfish only started recently but has increased exponentially over the last few years
80 as the species is increasingly being used as cleaner fish to control parasitic sea lice
81 in salmon farming (Powell et al., 2018a). Different lumpfish populations have
82 different fecundities and size at maturation (Whittaker et al., 2018), but an analysis
83 identified egg degumming as a potential way of increasing hatching success and

84 minimizing the risk of infectious diseases during embryo development (Powell et al.,
85 2018a). Under natural conditions, lumpfish eggs become sticky following exposure to
86 divalent calcium and magnesium in seawater (Lønning et al., 1984). Following
87 fertilization, males create funnel-like depressions in the egg mass and puff water and
88 vigorously fan the eggs to facilitate gaseous exchange and the removal of
89 nitrogenous waste, such paternal care being maintained throughout the incubation
90 period (Powell et al., 2018b). In contrast, lumpfish eggs in hatcheries may be left to
91 incubate in large clumps or shaped into a thin monolayer and reared in upwelling
92 incubators (Powell et al., 2018a). Hatching success is very variable in culture and it
93 is not uncommon to lose entire egg batches due to poor oxygenation and
94 subsequent spread of bacterial and fungal diseases (Powell et al., 2018a). Since a
95 female can typically produce in excess of 100,000 eggs (Davenport, 1985; Powell et
96 al., 2018b), even a modest increase in hatching rate would yield a significant
97 improvement in the number of larvae produced.

98 As with ballan wrasse (Grant et al., 2016), a small-scale pilot trial showed
99 recently that alcalase could also be effective at degumming lumpfish eggs (Powell et
100 al., 2018a), but the side effects and most efficient concentrations were not
101 investigated. Thus, the aim of this study was to test the potential benefits of alcalase
102 treatment to remove the adhesive layer of the lumpfish eggs and examine any
103 potential impacts on embryo survival, timing of hatching, growth, and incidence of
104 malformations. To this end, two experiments were conducted, one under controlled
105 laboratory conditions, where accurate counts could be made of daily mortalities in
106 replicated mini-incubators, and one under commercially relevant conditions in order
107 to validate the results obtained in the laboratory.

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110 **2. Materials and Methods**

111 *2.1. Source of eggs and artificial fertilization*

112 Wild lumpfish were obtained from commercial fishermen operating in Dorset (50° 44'
113 N; 2° 20' W) and Guernsey (49° 27' N; 2° 31' W) during 2016 and 2017 and were
114 transferred to a quarantine unit at the Centre for Sustainable Aquatic Research
115 (CSAR, Swansea University) where they were kept in 1.5 m³ circular tanks at 32 ppt
116 salinity, 13°C ± 0.3°C, and 8:16 L:D photoperiod. Broodstock were fed a mixture of
117 frozen blue mussels, squid, krill and whitebait.

118 Eggs were stripped from anaesthetized females (2-phenoxyethanol,
119 concentration 0.03 ml/litre) and milt was collected from euthanized males as
120 described in Powell et al. (2018a). Sperm motility was assessed by activation of a
121 small sample with seawater, followed by observation under the microscope at x40
122 magnification. Sperm was diluted with a milt extender (AquaBoost SpermCoat,
123 Cryogenetics AS, Norway) at a 1:1 ratio and maintained refrigerated at 4°C for up to
124 14 days. Artificial fertilization was carried out by adding 2-3 ml of extended milt to a
125 batch of eggs (~600 g), followed by gentle agitation for 1 min, activation with ozone-
126 treated seawater, further agitation for 2 min, and rinsing with clean seawater. Egg
127 hardening was completed after c. 30 min at 10°C.

128

129 *2.2. Laboratory (in vitro) degumming trial*

130 Three liquid alcalase (*Bacillus licheniformis*; 126741; VWR, UK) solutions were
131 prepared using 0.2 µm filtered seawater as a diluent to achieve concentrations of
132 0.02%, 0.2% and 2.0% along with a control treatment (0% alcalase). These were
133 maintained in the dark at 4°C for a maximum of 48h prior to use. Immediately

134 following sperm activation, and prior to egg hardening, 12 batches of fertilized eggs
135 ($n = 104 \pm 10$ eggs) from a single female were exposed for 5 min to one of the four
136 alcalase concentrations, gently inverting the vial to ensure uniform exposure for all
137 eggs. Following alcalase exposure, eggs were allocated at random to 12 x 0.5 L
138 upwelling vertical incubators constructed from 750 mL plastic funnels fitted with a
139 500 μm mesh “cradle” to hold the eggs. Flow was maintained at 1 Lmin⁻¹ (± 0.05 L),
140 and water temperature was maintained at 8.0 °C \pm 0.1 °C during the 37-day duration
141 of the trial. Eggs were disinfected with 30 ppm Pyceze (Novartis Pharmaceuticals UK
142 Ltd) for 20 minutes every 5 days.

143 Eggs were inspected 48 h post-fertilisation (morula stage) to assess their
144 viability and estimate fertilization success (Powell et al., 2018b). Degumming
145 efficiency was assessed by counting the number of single eggs (i.e. those not in a
146 clump, bound together by their adhesive chorion) and dividing by the total number of
147 eggs in the sample. They were then photographed using a Leica DFC290 digital
148 camera (Leica Microsystems Ltd, UK) mounted to a Nikon SMZ 800 Zoom
149 Stereomicroscope at X20 magnification. Mean chorion thickness (± 10 μm) was
150 obtained from photomicrographs of six eggs per replicate ($n=18$ per treatment) at 2-4
151 equidistant points of the egg, as described in Songe et al. (2016).

152 Hatched larvae were collected daily from individual incubators and preserved
153 in 10% formalin (HT501128; Sigma-Aldrich, UK) to screen for embryo malformations.
154 Hatching success was calculated by dividing the number of hatched larvae by the
155 number of viable eggs (i.e. excluding non-viable eggs that failed to develop after 24
156 hrs). A sample of fixed larvae ($n=6$ per replicate, $n=18$ per treatment) were
157 photographed and the standard length was calculated from the tip of the snout to the
158 caudal peduncle (resolution $\pm 10\mu\text{m}$). The incidence of embryo deformities such as

159 spinal scoliosis (Gapasin et al., 1998; Merchie et al., 1997), and operculum and
160 sucker deformities was recorded.

161

162 *2.3. Degumming under commercial conditions*

163 A 2% alcalase concentration was chosen for the large commercial trial, as this had
164 shown promising results in a small-scale pilot study (Powell et al., 2018a). A solution
165 was prepared by adding 10 ml of alcalase to a 20-litre beaker containing 490 ml of
166 filtered sea water. Fertilised lumpfish eggs (590 ± 10 g) were added to the beaker
167 and aerated. For the control treatment, the same amount of eggs was added to 500
168 ml of filtered sea water. After 5 minutes, eggs were rinsed with 1 litre of filtered sea
169 water and transferred to 70 litre hopper incubators with a 20 L/min upwelling flow
170 and a 500 μ m screen mesh to keep the eggs off the bottom. Salinity was maintained
171 at 32 ppt and water temperature at 9.0 ± 2.0 °C. Eggs were treated with Pyceze
172 every five days, as above. Approximately 30 eggs were sampled from each clump
173 using a grid system to ascertain viability and degumming efficiency, as described
174 above. During the peak of mass hatching, 100 larvae per batch were reared in
175 triplicate in 30 litre tanks and monitored for eight weeks under the same commercial
176 conditions (temperature 9.0 ± 2.0 °C) and fed enriched *Artemia* and dry diet.
177 Mortalities were recorded daily for each replicate.

178

179 *2.4. Statistical analysis*

180 We used R v3.4.1 (R Core Team, 2017) for statistical analysis. Degumming
181 efficiency, survival, and incidence of embryo malformations (all proportion data) were
182 analysed via Generalized Linear Mixed-Effect Modelling (GLMM) using the R
183 package “*lme4*” (Bates et al., 2015) and a binomial error distribution, with alcalase

184 concentration as a fixed factor and incubator ID as a random effect. Hatching
185 success was modelled as a function of time and alcalase concentration as
186 predictors, and incubator ID as a random effect, using the packages “*survival*”
187 (Therneau, 2015) and “*FrailtyPack*” (Rondeau et al., 2012) to account for
188 unmeasured heterogeneity. The effect of alcalase concentration on chorion
189 thickness and larvae size at hatching was tested by Linear Mixed Modelling (LMM)
190 using alcalase concentration as a fixed effect, and incubator identity as a random
191 effect using the *lme4* and *lmerTest* (Kuznetsova et al., 2017) R packages. We
192 compared models using untransformed and log₁₀ transformed alcalase
193 concentrations, and also treating alcalase concentration as a continuous or discrete
194 predictor. Inspection of AIC values indicated that treating alcalase concentration as a
195 factor resulted in the most plausible models.

196

197 *2.5. Ethics Statement*

198 This study adhered to the ARRIVE guidelines and was approved by Swansea
199 University Animal Welfare Research Body (permit IP-1516-16)

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203 3. Results

204 3.1. Efficacy of alcalase as a degumming agent

205 Alcalase was effective at separating (degumming) lumpfish eggs at concentrations of
206 0.2% and 2% for 5 minutes, but not at 0.02% (Figure 1, glmer: estimate = 1.97, SE =
207 0.226, Z value = 8.724, $P < 0.001$). Compared to controls, eggs exposed to alcalase
208 concentrations of 0.2 and 2% displayed mean degumming rates of 61% (± 12) and
209 75% (± 21), respectively. The degumming effect of alcalase was confirmed by
210 measurements of chorion thickness, which decreased significantly with increasing
211 alcalase concentration (Figure 2; lmer; $F_{3,11.852} = 11.102$, $P < 0.001$). Thus,
212 compared to controls (mean chorion thickness = $68.59 \pm 2.91 \mu\text{m}$), chorion thickness
213 decreased by $8.77 \mu\text{m}$ (± 1.91) at 0.2% and by $10.24 \mu\text{m}$ (± 1.93) at 2% alcalase.

214

215 3.2 Effect of alcalase on hatching rate and timing of hatching

216 There was no significant difference in hatching success between controls and eggs
217 exposed to alcalase, once tank effects were taken into account (Figure 3; frailty
218 model treatment estimate = -0.084, SE = 0.056, Z = -1.48, $P = 0.138$; Exponential
219 distribution

220 $\text{Loglik}(\text{model}) = -2801.9$, $\text{Loglik}(\text{intercept only}) = -2806.4$; $\chi^2 = 8.95$, $\text{df} = 4.2$, $P =$
221 0.07). However, alcalase treatment resulted in a shorter hatching period compared to
222 controls ($F_{3,754} = 69.55$, $P < 0.001$), decreasing mean hatching time by -0.66 days (\pm
223 0.11) in the case of 0.02% alcalase ($t = -5.974$, $P < 0.001$) and by -1.59 days (± 0.11)
224 in the case of 2% alcalase ($t = -14.097$, $P < 0.001$).

225

226 3.3 Effect of alcalase on larvae size and incidence of malformations

227 Embryo size increased with increasing alcalase concentration (Figure 4, lmer:
228 estimate = 53.99, SE = 19.27, $t_{69.03} = 2.80$, $P = 0.006$). Compared to controls, eggs
229 treated with 2% alcalase resulted in embryos that were $148.14 \pm 44.76 \mu\text{m}$ larger
230 ($t_{68.98} = 3.31$, $P = 0.001$). No deformities were observed under the microscope in any
231 of the larvae from any treatment.

232

233 3.4. Effects of alcalase under commercial conditions

234 Alcalase was also effective at degumming lumpfish eggs under large scale,
235 commercial conditions (estimate = 0.531, SE = 0.230, Z value = 2.306, $P < 0.05$).
236 Exposure of fertilised eggs to 2% alcalase for five minutes resulted in a mean
237 degumming rate of 0.70 ± 0.06 , compared to 0.0 for controls. Alcalase treatment had
238 no significant effect on larvae survival eight weeks post-hatch (estimate = -0.2285,
239 SE = 0.1782, $t = -1.282$, $P = 0.2$), but eggs exposed to 2% alcalase were significantly
240 larger (mean = $14.4 \text{ mm} \pm 0.14$) than controls (mean = $13.3 \text{ mm} \pm 0.15$; $F_{1,239} =$
241 41.62 , $P < 0.001$), confirming the results of the *in vitro* laboratory trial.

242

243 3.5. Cost benefit analysis

244 During the commercial trial, we exposed fertilised lumpfish eggs at a ratio of one egg
245 to one millilitre of alcalase solution. The alcalase solution was purchased at a cost of
246 £46.45 for 500ml, but placing a large bulk order can probably obtain it c. 25%
247 cheaper at £74.3 per Litre. Based on this bulk cost, and £10,000 fixed costs for the
248 sourcing of wild broodstock, we estimated the benefit to cost ratio of incubating 1
249 million lumpfish eggs under different egg market prices (1-8 pence/egg), taking into
250 account different hatching and degumming rates at different alcalase concentrations.
251 The simulations (Figure 5) suggest that the optimal alcalase concentration is 0.2%

252 (over five minutes), and that benefits outweigh costs at 1.56 pence per egg which is
253 the break even point.

254

255 **4. Discussion**

256 Without parental care in hatcheries, adhesive eggs tend to display reduced gaseous
257 exchange rates, and this can make them more susceptible to opportunistic
258 pathogens (Demska-Zakes et al., 2005). Our results show that alcalase can be
259 effective at removing the adhesive layer of the lumpfish chorion, and that treated
260 eggs result in larger larvae, without any evidence of adverse effects on larval survival
261 or embryo malformations. These findings are similar to work carried out on ballan
262 wrasse, where alcalase treatment achieved >69% degumming rate on a dosage-
263 dependent fashion without any detrimental effects (Grant et al., 2016).

264 The advantages of egg degumming are various. Degummed eggs allow for
265 more thorough disinfection, easier handling, more accurate egg counting and
266 estimation of fecundity and survival rates, and potentially also more efficient use of
267 space for egg incubation. This can in turn increase hatching success, reduce health
268 risks, and allow for more efficient commercial production (Linhart et al., 2000; Linhart
269 et al., 2003).

270 Our results indicate that although a five-minute exposure to 2% alcalase
271 achieved the highest degumming rate, a concentration of 0.2% achieved similar
272 results and can be more cost effective, based on a cost-benefit analysis. Little
273 degumming, on the other hand, was observed at a concentration of 0.02% alcalase,
274 although it is possible that increasing exposure time over 5 minutes may achieve
275 better degumming rates, as shown for ballan wrasse (Grant et al., 2016). We
276 exposed eggs to alcalase within ten minutes of artificial fertilisation, before the eggs

277 had hardened. Further research into the feasibility of degumming naturally spawned
278 (hardened) eggs may be warranted, as naturally spawned clumps of eggs are often
279 encountered in culture (Treasurer et al., 2018).

280 One unexpected consequence of alcalase treatment was a faster embryo
281 development time and larger larvae. Although the reasons for this are unclear, it is
282 possible that the thinner chorion of degummed eggs may have increased oxygen
283 availability and allowed for more efficient use of energy. Oxygen availability is
284 known to affect egg quality and embryo development in carp (Vodianitskiy et al.,
285 2017), and in general increased oxygen results in larger embryos for many fish
286 species (Braga Goncalves et al., 2015). Embryos developing within thick gelatinous
287 egg masses often show retarded development in marine gastropods (Chaffee &
288 Strathmann, 1984), and it is possible that the same may happen in lumpfish.
289 Whatever the reasons, a significant size advantage was evident 8 weeks post-
290 hatching, even though degummed eggs only hatched less than 2 days earlier than
291 controls. The larger size of larvae originating from degummed eggs could be
292 beneficial to industry, as larger larvae have a greater probability of surviving past the
293 critical period during the transition from endogenous to exogeneous feeding (Garrido
294 et al., 2015).

295 Although a thick egg chorion can make it more difficult for water moulds, such as
296 *Saprolegnia*, to penetrate the eggs (Songe et al., 2016), it can also favour settlement
297 of water borne particulates that facilitate fungal and bacterial infection and can make
298 eggs more vulnerable. Indeed, an adhesive chorion is thought to be particularly
299 susceptible to viral, fungal and bacterial infection, and one of the benefits of
300 achieving a thinner chorion through chemical degumming may be to reduce
301 pathogen loads and facilitate egg prophylaxis under aquaculture conditions (Krise et

302 al., 1986). Egg degumming in lumpfish could also make it possible to use various
303 types of upwelling incubators that maintain eggs in suspension, such as McDonald-
304 type jars, pelagic egg jars, and Imhoff cones (i.e. Jensen et al., 2008). Their use
305 could achieve a more uniform distribution of clean, oxygenated water with less
306 chance of infection by water moulds. This is an area that warrants further attention
307 because growth of opportunistic bacteria and water moulds on dead eggs can
308 spread quickly and affect surrounding viable eggs (Davenport, 1983). Removing the
309 adhesive layer can help to alleviate this problem as it would enable easier separation
310 and removal of non-viable eggs. In this sense, several techniques are available for
311 removing and counting eggs, including manual siphoning, use of flotation methods
312 (i.e. Leitritz & Lewis, 1976) and use of automatic egg sorters, which could prove
313 effective at separating large numbers of eggs if these are degummed and no longer
314 form clumps. For example, using the method described by Coombs (1981), viable
315 and non-viable lumpfish eggs could be separated using a column and a salinity
316 gradient.

317

318

319 **Conclusions**

320 In summary, we describe (and validate under commercial conditions), a method for
321 chemically removing the adhesive layer of lumpfish eggs immediately after artificial
322 fertilization using the enzyme alcalase. A five minute exposure to 2% alcalase
323 resulted in 70% degumming rate, 14% reduction in chorion thickness, and larger
324 larvae without impacting on hatch rates or larvae survival up to 8 weeks after
325 hatching. Cost-benefit analysis indicates that the costs of alcalase treatment are
326 offset under most conditions and that a 0.2% concentration is the most cost-

327 effective. Egg degumming can facilitate the separation and removal of non-viable
328 eggs, improves egg husbandry, and provides scope for using modified incubators
329 that can reduce incubation space and decrease labour time compared to current
330 methods.

331

332

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340

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487 **Figures**

488 Figure 1. Effect of a 5-minute exposure to different alcalase concentrations on
489 proportion (mean \pm 95% CI) of degummed lumpfish eggs

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491 Figure 2. Effect of alcalase treatment on thickness of the egg chorion. Boxplots show
492 minimum, first quartile, median, third quartile, and maximum values. Extreme values
493 are shown by closed circles.

494

495 Figure 3. Effect of alcalase treatment on hatching success and timing of hatching
496 (cumulative proportion of hatched larvae).

497

498 Figure 4. Effect of alcalase treatment on size of lumpfish larvae (standard length).
499 Shown are minimum, first quartile, median, third quartile, and maximum values.
500 Extreme values are shown by closed circles.

501

502 Figure 5. Benefit-cost analysis for the production of 1 million lumpfish eggs using
503 different alcalase concentrations and various egg sale prices (GBP pence/egg).
504 Dotted line represents the estimated break-even point (1.56 pence/egg).

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